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Visualization of neuronal geometry in ventral nerve cord ganglia of Acheta domesticus

Gordon William Luke
University of the Pacific

Visualization of Neuronal Geometry in Ventral Nerve Cord Ganglia of *Acheta domesticus*

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Master of Science

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by

Gordon William Luke

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This thesis, written and submitted by

GORDON WILLIAM LUKE

is approved for recommendation to the Committee on Graduate Studies, University of the Pacific.

Department Chairman or Dean:

F. R. Hunter

Thesis Committee:

June Fouldhouse Chairman

Dallas McCleaf

Alice S. Hunter

Dated 24 March 1976

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INTRODUCTION

In the common field cricket, Acheta domesticus, the ventral nerve cord consists of the subesophageal ganglion and three thoracic ganglia, followed by five smaller abdominal ganglia. The subesophageal ganglion is considered to be the fusion of three ganglia that control the organs of the lower portion of the head, specifically the labial palps, salivary glands, and the general area of the neck (Callahan, 1971).

The first thoracic ganglion gives off four pairs of nerves and innervates the muscles and hypodermis of the prothorax as well as the forelegs. The second thoracic ganglion is slightly larger than the first and also has four pairs of nerves. This ganglion supplies innervation to the middle pair of legs and to the muscles and hypodermis of the mesothorax. The third thoracic ganglion is the largest in the cricket body and is heart-shaped in appearance (Fig. 1). It gives off four pairs of thoracic nerves and six pairs of abdominal nerves. The thoracic nerves supply the muscles and hypodermis of the metathorax and also the large muscles of the hind jumping legs (Albrecht, 1953). The abdominal nerves innervate the first three segments of the abdomen. The abdominal innervation from this ganglion, Albrecht (1953) and Callahan (1971)

concluded, also includes the first three abdominal ganglia, which have fused.

The fourth, fifth, sixth, and seventh abdominal ganglia give off nerves which supply abdominal segments four through seven. The eighth, or terminal ganglion of the abdomen, is slightly larger than the previous abdominal ganglia and innervates the eighth abdominal segment, the external reproductive organs, and the paired cerci (Albrecht, 1953). All of the ganglia of the ventral nerve cord are joined by a pair of thick connectives known as interganglionic connectives. These interganglionic connectives consist of bundles of axons and their supporting cells (Callahan, 1971). The length of the connectives varies with the type of ganglion supplied. The abdominal ganglia connectives measure approximately 2.0 - 3.0mm whereas the short and thick pair between the second and the third thoracic ganglia measures approximately 1.0mm.

Although the nerve trunks themselves are relatively easy to visualize under the dissecting microscope, individual cell bodies, axons, and dendrites remain invisible because of the translucence of the nervous tissue. One of the older techniques used for observing individual neurons was the Golgi method (reduced silver) used effectively by Cajal. Although those neurons that are stained tend to fill entirely, it is estimated that only 10% of the neurons are stained and the results are often of poor quality (Pitman et al., 1972). More

recently, intracellular injection of procion yellow dyes has been used for the definition of neural structure. The major drawbacks of this method are that fine branches of nerve processes often remain unobservable in whole mount and the dye itself is not electron-dense so that ultrastructural study is impossible (Kravitz, 1968). Procion yellow also has the inherent weakness of poor migrational properties which necessitates the use of an electrical current (electrophoresis) (Kater et al., 1973).

The axonal iontophoresis method of Iles and Mulloney (1971) enables cell bodies and their processes to be filled with dye by electrophoresis along the axons of a severed nerve. Pitman et al., (1971) modified this method by substituting cobaltous chloride for procion yellow. The use of cobaltous chloride is advantageous because it fills the neurons uniformly and when precipitated with ammonium sulfide it forms an electron-dense compound (cobalt sulfide) within the neuron. This technique has proved useful for electron microscopy as well as for light microscopy. It has been reported (Pitman et al., 1972) that the chlorides of copper, iron, and nickel should also be possible to use for iontophoresis. I have further modified the axonal iontophoresis technique by not applying any form of electrical current, thus allowing the nerve to act as a "wick" (after Mason, University of California, Berkeley, personal communication).

The present study is an attempt to show the pattern of

innervation, or neuronal geometry, of various nerves leading to their respective ganglia (Fig. 1) and to obtain visualization of the finest terminal branches. A description of the cellular arrangement of a segmental ganglion would be of value in better understanding the results obtained when using the axonal iontophoresis method applied to the third through seventh ventral nerve cord ganglia. A segmental ganglion of the thorax or abdomen is usually an oval mass of nerve tissue, continuous with the interganglionic connectives (Fig. A). Two or three principle lateral nerves extend from its sides. The ganglion is encased in a nucleated sheath, the neurilemma, which forms a continuous covering over the nerves and the connectives. The main cellular components of the ganglion, ganglion cells, are arranged peripherally, mostly in the lateral and dorsal parts. The central and ventral parts are occupied by a neuropil mass. The lateral nerves of the ganglion contain both motor and sensory fibers which arise from the dorsal and ventral roots, respectively, within the ganglion (Albrecht, 1953).

Five regions may be distinguished within the neuropil (Fig. B). Dorsally, the region of the dorsal interganglionic connective fibers may be seen. Just beneath this is the region of the dorsal nerve roots (motor center) while ventrally is situated the region containing the ventral connective fibers and immediately above these is the location of the sensory center, or region of the sensory ventral roots of the lateral

nerves. The central portion of the ganglion contains the neuropil mass. At the end of the ganglion, dorsal and ventral tracts continue into the connectives (Albrecht, 1953).

Snodgrass (1953) showed that each ganglion contains six groups of nerve elements: (1) cell bodies and roots of the motor fibers of the lateral nerves; (2) roots of the sensory fibers of the lateral nerves; (3) cell bodies and fibers of the interganglionic neurons; (4) cell bodies and collateral branches of the interganglionic neurons; (5) cell bodies and roots of the motor fibers of the median nerve, if present, and (6) roots of the sensory fibers of the median nerve, if present. The neuropil contains fiber endings from the lateral sensory nerves of the ganglion in question and also from ganglia situated more posteriorly along the nerve cord (Fig. B).

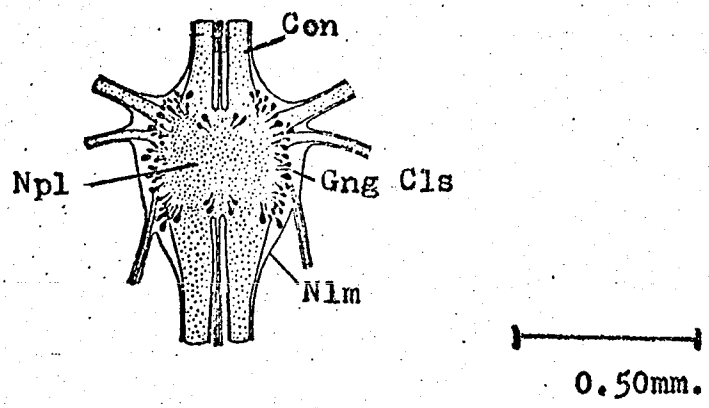
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Fig. A

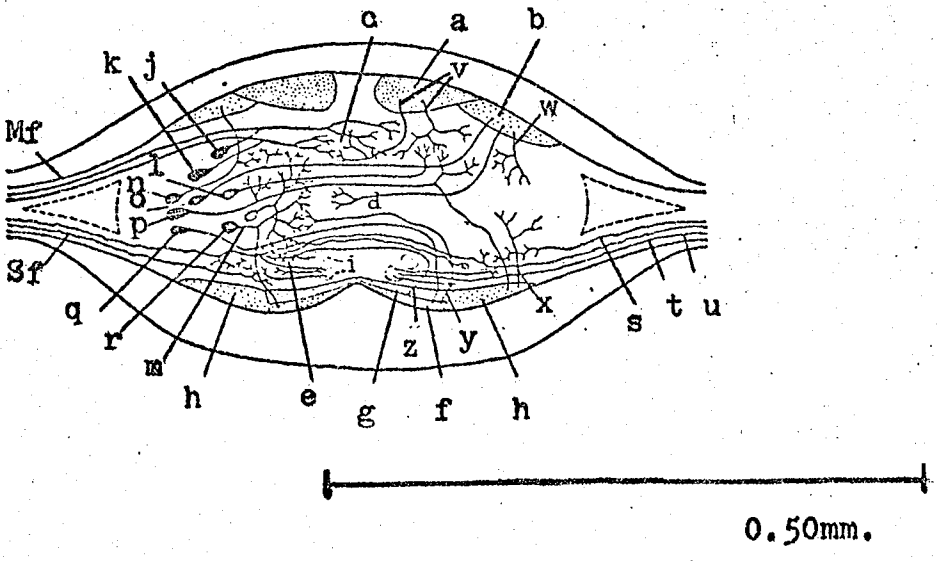
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Fig. B

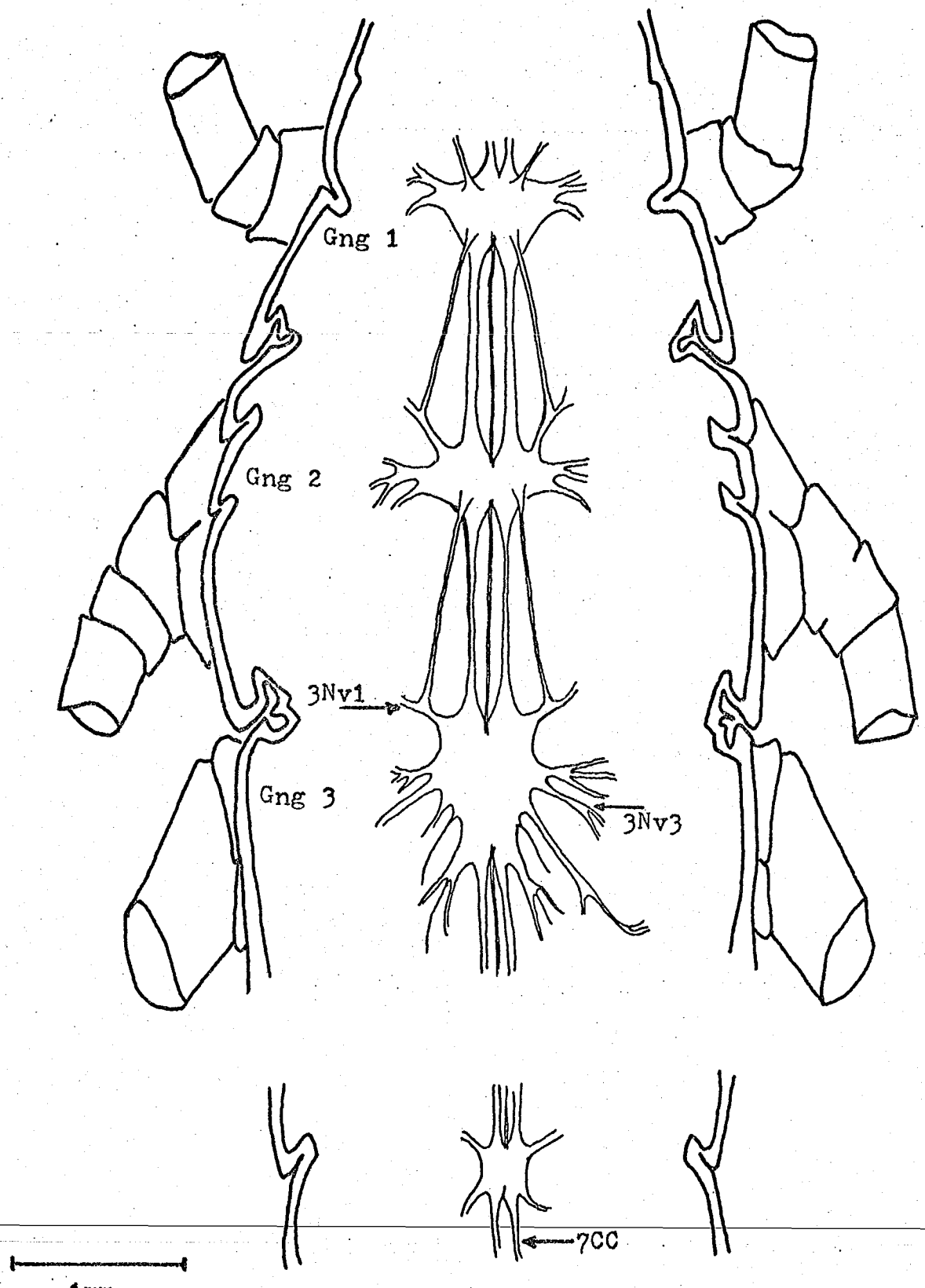

Fig. 1

MATERIAL AND METHODS

Adult male and female crickets of the species Acheta domesticus were obtained from laboratory stock. Individuals were chosen according to sexual maturity and size (over 2.5 cm in length). Using microdissecting scissors, both the legs and wings were removed to reduce any movement. Care was taken so that as little surrounding tissue as possible was damaged. The insect was then secured to the dissecting dish, ventral surface facing upwards, using heat-softened paraffin. The sternal plate covering the third thoracic ganglion was removed and the tissue surrounding the ganglion was dissected out. The tracheal supply to the ganglion was left intact in order to improve viability.

The nerve to be filled was freed of trachea and excess fat and moisture were removed. The nerve was then cut as far as possible from the ganglion to be studied and allowed to partially dry. These manipulations were carried out using small diameter wooden dowels fitted with size 0 insect pins. Petroleum jelly was applied to the entire surface surrounding the cut nerve using a syringe modified by grinding the end of the needle flat so that the aperture delivered a uniform bead. Petroleum jelly was used to carefully seal around the cut nerve so that leakage of cobaltous chloride onto the ganglion would be avoided. Using the same needle and syringe, a petroleum jelly bowl was fashioned around the nerve and was

made approximately 4 mm in height and 3-4 mm in diameter in order to hold 1-2 drops of cobaltous chloride. Since the entire dissection was done without the use of insect saline, the cut nerves tended to dry quickly and seal at the ends. A drop of distilled water was placed on the end of the cut surface of the nerve in order to reopen these sealed ends and cause the axons to swell. The tip of the nerve was then recut while in the distilled water so that the opening of the axons might further be assured (Mason, 1973).

After 3 minutes, the distilled water was drawn off and replaced with a drop of 50 mM to 250 mM cobaltous chloride. All remaining exposed tissue, except spiracles, was then covered with petroleum jelly to avoid desiccation. The dissecting dish containing the preparation was filled with small cotton swabs soaked in saline in order to provide a humid environment. The dish was covered and placed overnight in an incubator at 20°C.

After 12-24 hours, the remaining cobaltous chloride within the jelly bowl was precipitated using 0.05 ml ammonium sulfide (44%) in 5 ml of insect saline. By delivering a drop of this solution into the bowl, all remaining cobalt was precipitated and thus prevented from reacting with exposed tissues when the preparation was dissected from the cricket's body. The petroleum jelly bowl was eliminated using probes and the ganglion and attached filled nerve were re-

moved and placed in insect saline where all remaining petroleum jelly, fat body, and air sacs were removed. The preparation was placed in the 44% ammonium sulfide solution for 15 minutes to precipitate the cobalt. The preparation was then fixed in Carnoy's fixative for an additional 15 minutes, dehydrated in ethanol, and cleared and stored in methyl benzoate.

Axonal iontophoresis was performed on the following nerves (Fig. 1): third thoracic nerves I and III, filled toward the ganglion (proximally); the seventh abdominal ganglion interganglionic connectives (posterior pair), filled proximally and simultaneously. Preparations were studied and photographed in whole mount.

RESULTS

During my research, approximately 60 dissections were performed, 15 of which were performed on the brain-retrocerebral neuroendocrine system. Most of the preparations involving the brain-retrocerebral neuroendocrine system were largely unsuccessful because of the extremely small size of the nerves involved in this complex within the cricket and also because of the mobility of the head which made dissection difficult. Of the remaining preparations, the most satisfactory were those done towards the end of the research period when my skills and knowledge were most advanced. When problems of leakage are solved and when the proper concentrations of cobaltous chloride and ammonium sulfide are found, a success rate of 80-90% and above is most likely obtainable.

A. Nerve 3Nv3

Precipitation of the iontophoresed cobaltous chloride within nerve 3Nv3, filled proximally, reveals a fine pattern of neurites and axonal tracts. The immediate and surrounding areas where the nerve trunk joins the ganglion is infiltrated with small, densely black fibers projecting into the ganglion itself (Fig. 2,3,4). One rather thick axonal tract, approximately 20 nm in diameter, is seen to enter the ganglion and then exit to the left through nerve 3Nv4, which also innervates the hind leg. The nerve trunk of 3Nv4 is situated

immediately to the left of 3Nv3 and appears light in tone (unfilled) except for the bundles of axons seen entering. An axonal bundle of the same diameter is seen to branch off of the tract entering 3Nv4 and lateral to it. A tract of perhaps 5 to 6 large individual axons extends downward into the ganglion from 3Nv3 and then descends out of the plane of focus. The nerve trunk itself appears densely black because of normal extracellular leakage of cobaltous chloride at the entrance of the cut nerve (site of initial filling). The darkened swirls to the right, near the border of the photomicrograph, are due to movements in the methyl benzoate preservative during photography. The dark areas around the axonal tracts and near the base of the nerve trunk (3Nv3) are due to masses of fine dendritic branches.

B. Nerve 3Nv1

This nerve is approximately 40 nm in diameter and was also filled proximally (Fig. 5,6,7). The trunk is relatively light in tone compared to other preparations and this may be due to poor migration of the cobaltous chloride solution, or little extracellular leakage. However, bundles of axons are visible near the midpoint of the nerve trunk and can be seen entering the ganglion. The pattern of innervation appears similar to that of 3Nv3 although there are fewer tracts visible and less branching is to be seen. The dark mass of tissue situated to the right of the nerve trunk is an abnormal tumor-like growth which was noticed in this

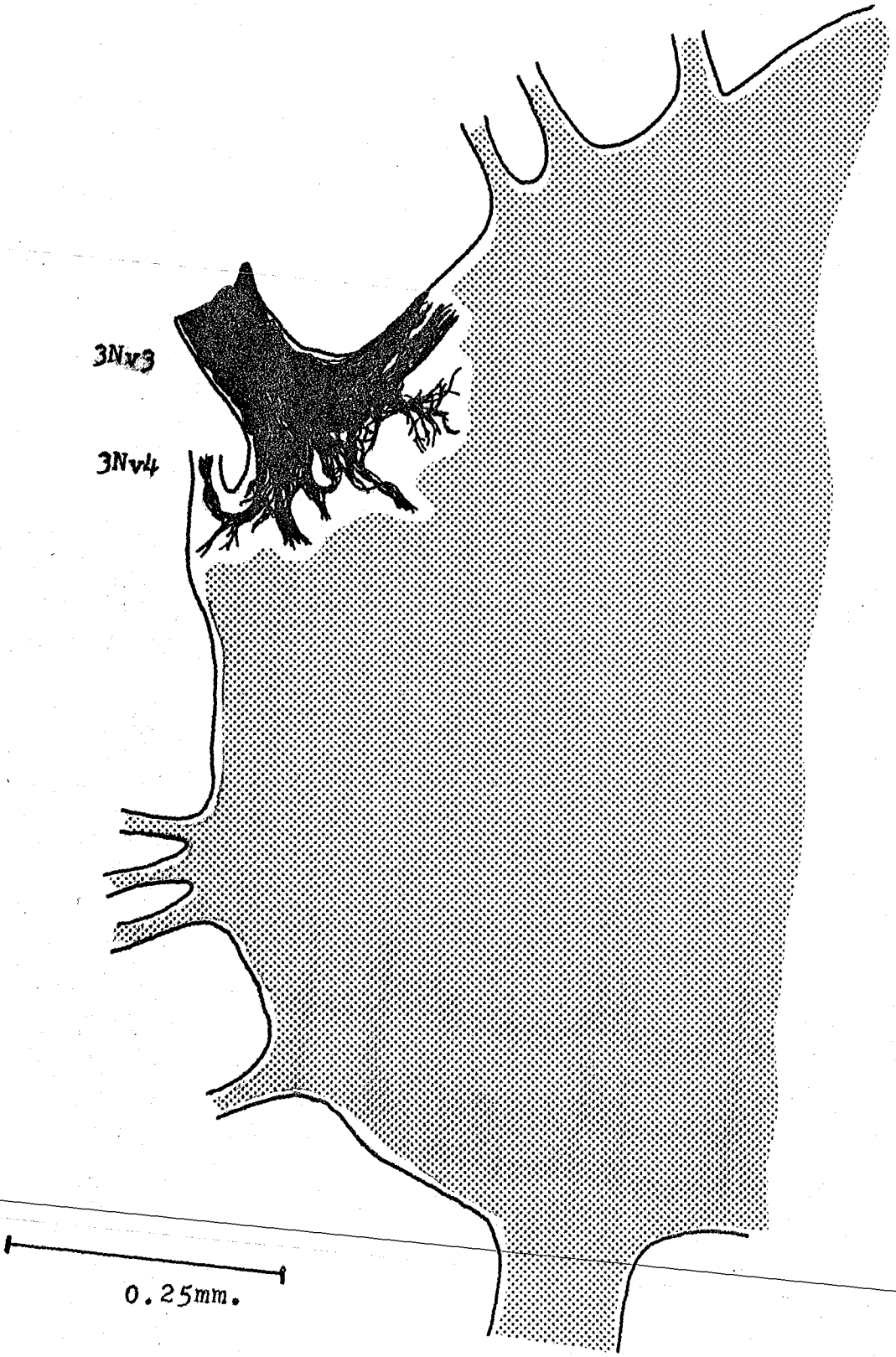

Fig. 2

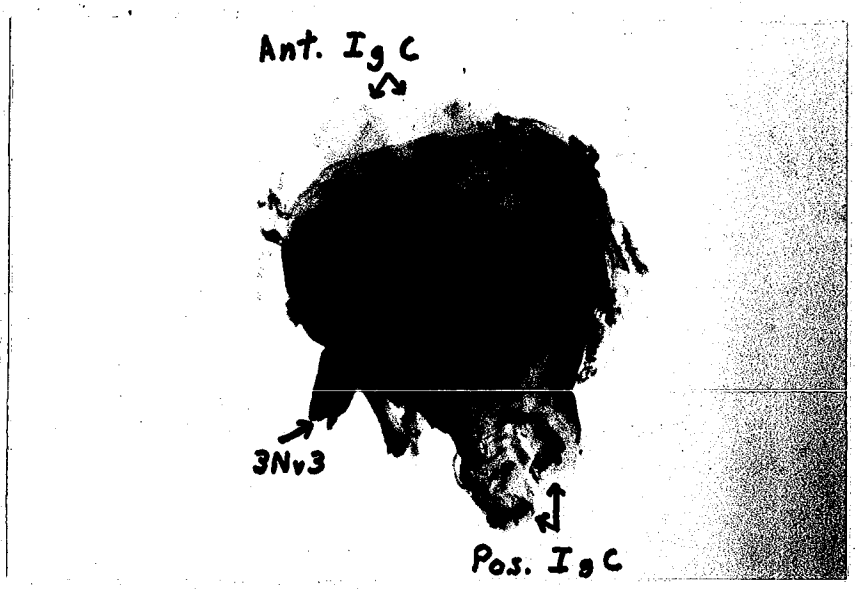

Fig. 3

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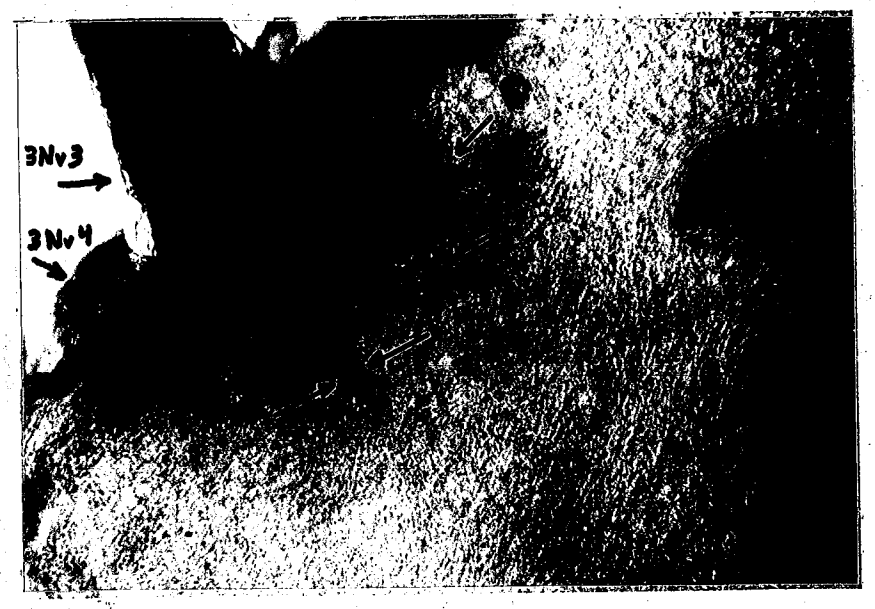

Fig. 4

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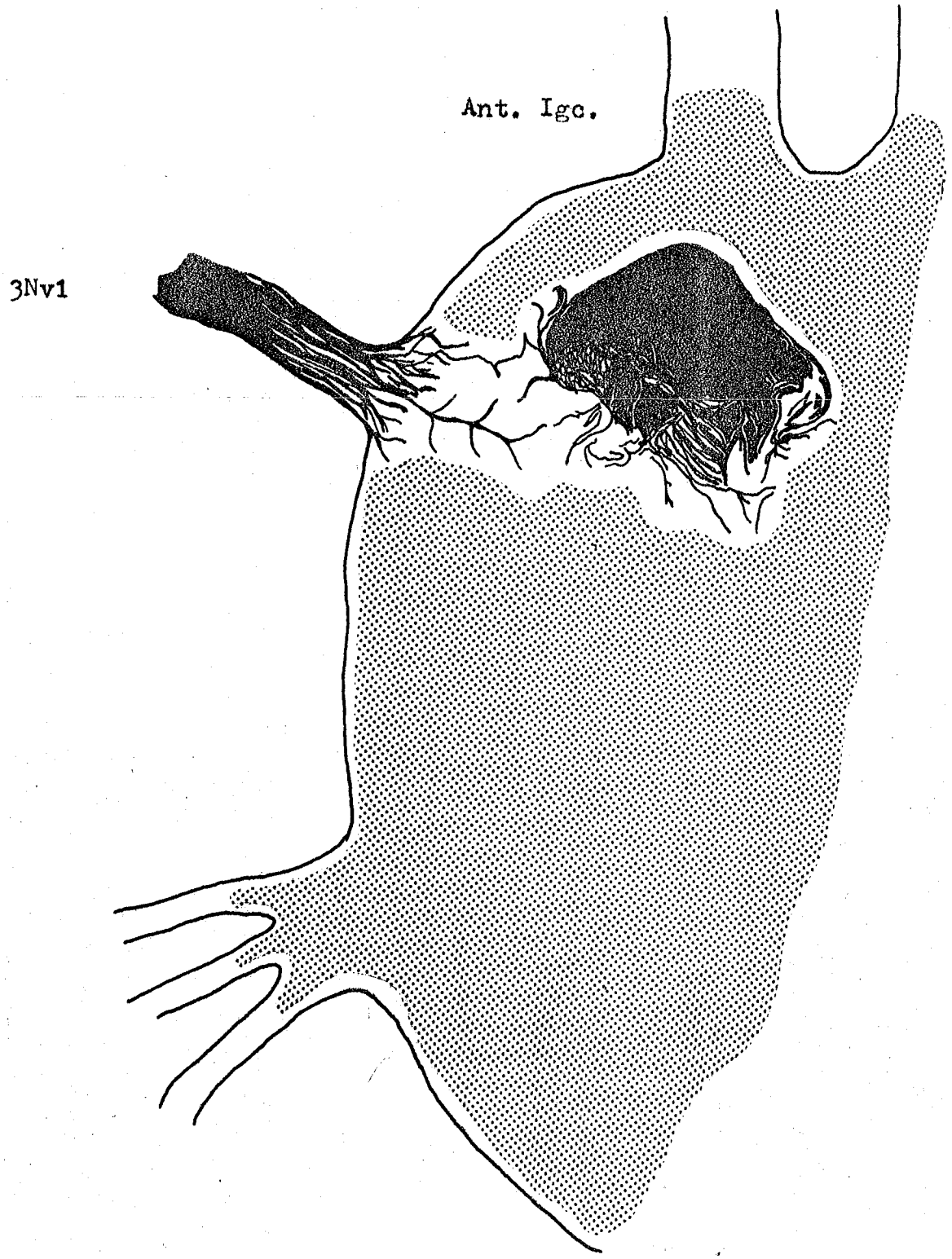

Fig. 5

0.25mm.

Fig. 6

1 mm

Fig. 7

0.5 mm

specific dissection. The mass was located slightly posterior to the anterior pair of interganglionic connectives and was light brown in contrast to the whiteness of the normal ganglion tissue surrounding it. The tissue mass became darker when exposed to ammonium sulfide.

C. Seventh abdominal ganglion

The posterior pair of interganglionic connectives, leading into the seventh abdominal ganglion, were filled proximally. These connectives measured approximately 75 nm in diameter at their thickest point and can be seen to be filled with fibers that become darker as they near the body of the ganglion and its neuropil (Fig. 8,9,10). The ganglion is dark in appearance because of its close proximity to the filling site and some extracellular leakage of cobalt. Fine fibers can be seen exiting the ganglion through the paired sternal nerves and also through a single and more lateral tergal nerve.

D. Sixth abdominal ganglion

This ganglion was filled from the same pair of connectives as the seventh abdominal ganglion (posterior connectives, seventh abdominal ganglion) and is not stained as darkly as was the seventh abdominal ganglion because it is 1.5 mm away from the site of filling and fewer axons have filled (Fig. 11,12). The opaque area located in the central portion of the ganglion is the neuropil and is a thick mass

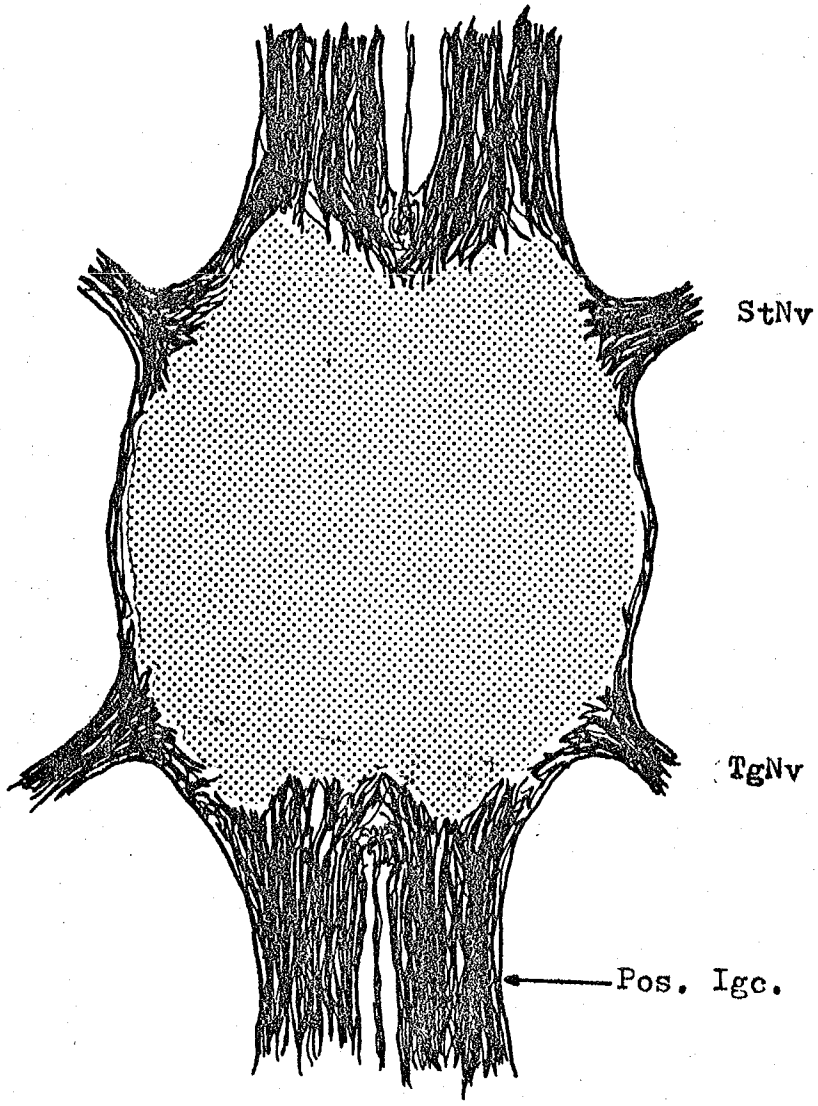

Fig. 8

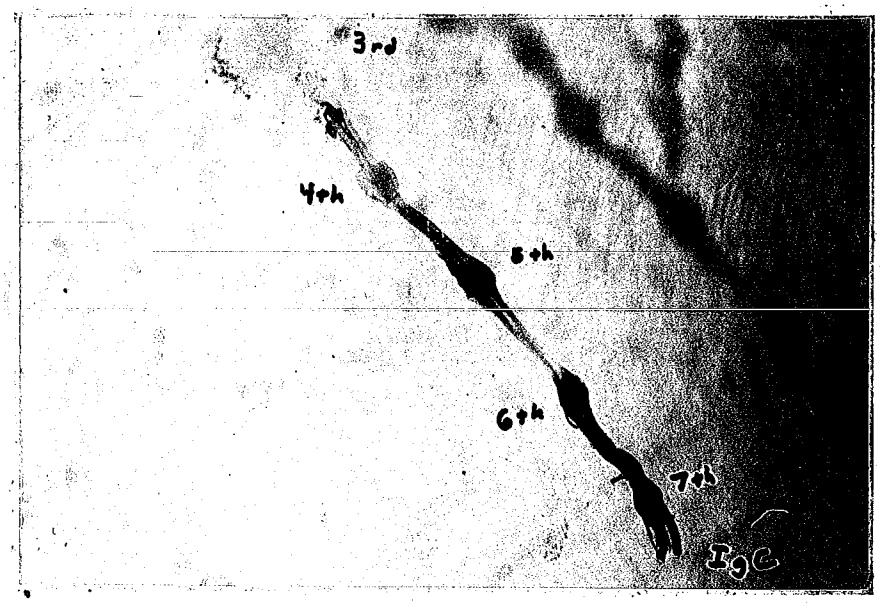

Fig. 9

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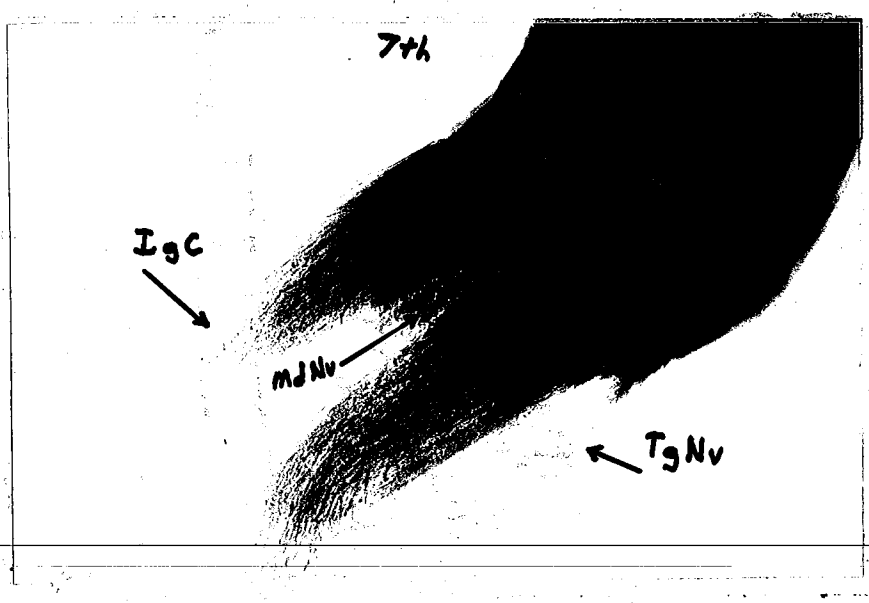

fig. 10

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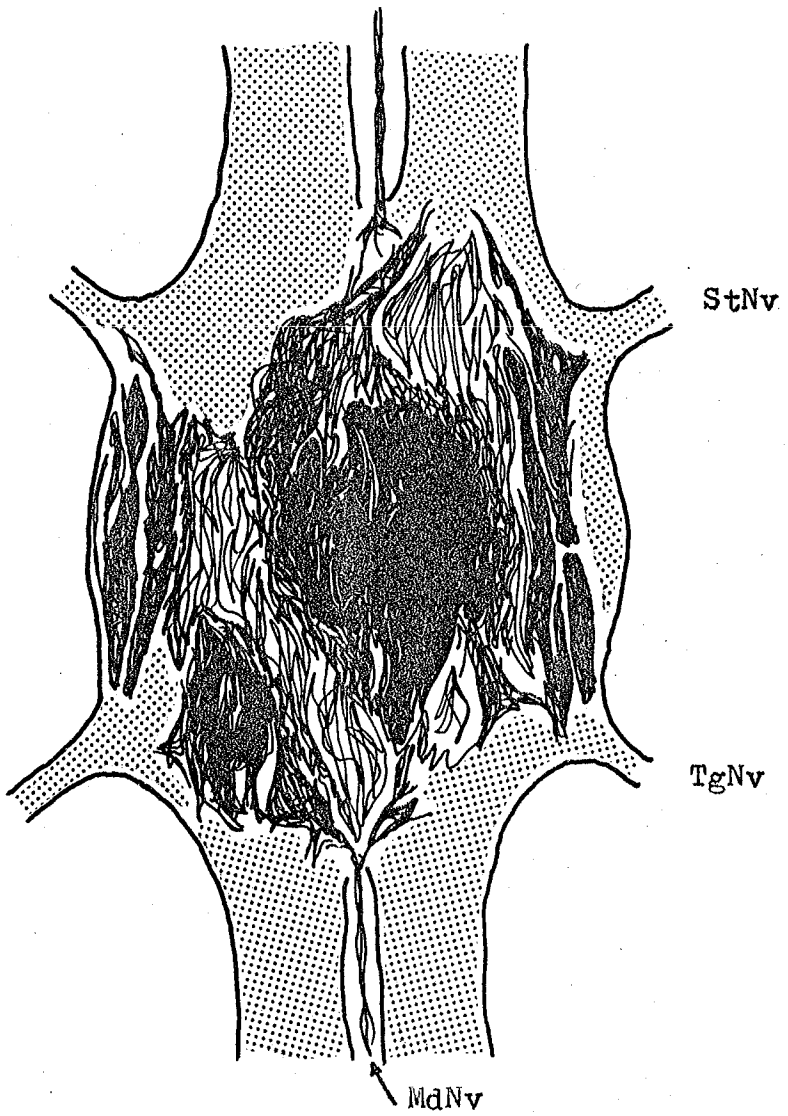

Fig. 11

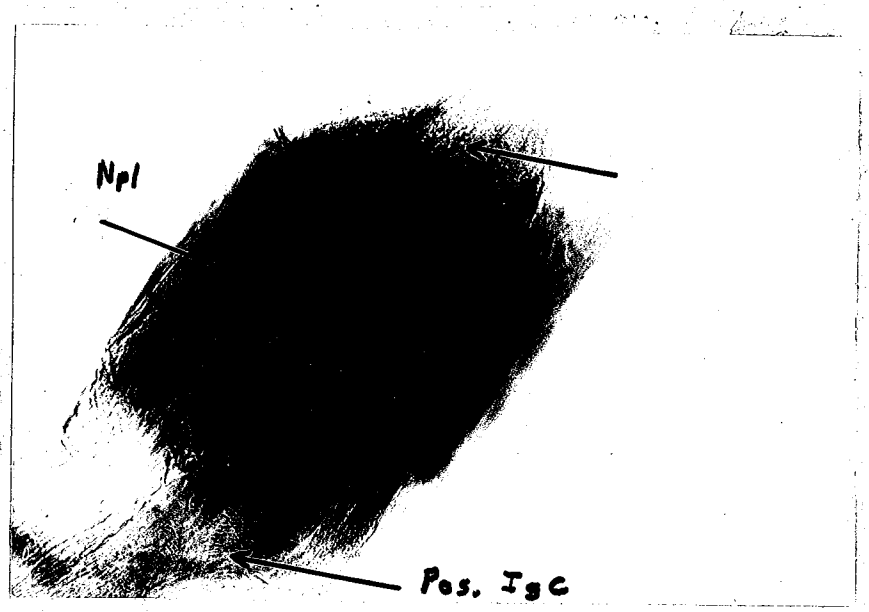

Fig. 12

0.5 mm

of fibers and nerve cell bodies, which are not visible here. A lateral nerve, probably a small tergal nerve, is seen to be filled with small fibers. Some individual fibers can be seen at either end of the ganglion near the interganglionic connectives.

E. Fifth abdominal ganglion

The fifth abdominal ganglion was filled via the posterior interganglionic connectives of the seventh abdominal ganglion as in the previous two preparations. It is lighter in appearance than either of the others because it is still further away from the initial filling site and as a result fewer fibers have filled. The neuropil is centrally located within the ganglion and many fibers and cell bodies are visible, although most remain out of the plane of focus. A small darkened area anterior and lateral to the midpoint of the neuropil contains two cell bodies. Directly opposite these cells, on the other side of the neuropil, is a row of seven cells that happen to be in the same focal plane. As usual, fibers are seen to enter the ganglion via the connectives (posterior pair) and exit through the anterior pair of connectives and continue into the next ganglion (Fig. 13,14).

The fourth abdominal ganglion did not fill appreciably and was not studied.

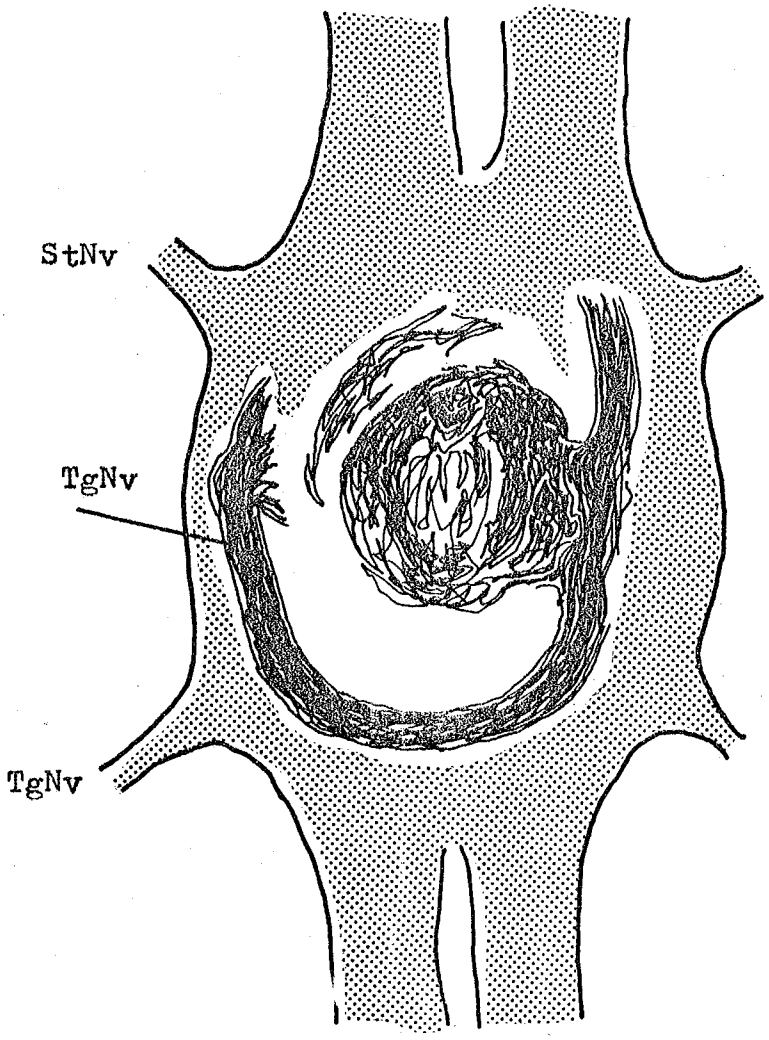

Fig. 13

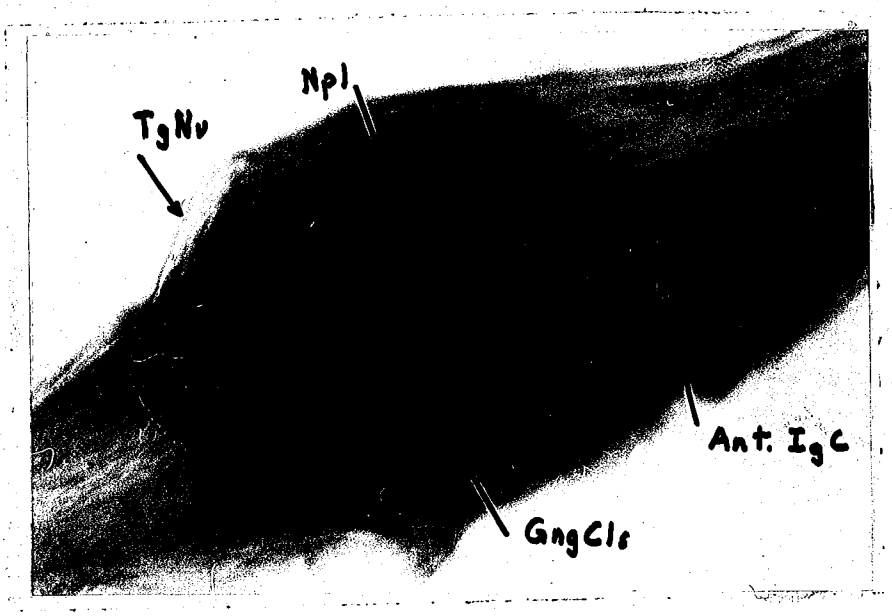

Fig. 14

0.5 mm

DISCUSSION

The technique used by Iles and Mulloney (1971) to move procion yellow into a neuron by iontophoresis via cut nerve trunks has also been shown to be effective with the substitution of cobaltous chloride. In this study, I have used this procedure, modified by the absence of an electrical current, to stain nerve fibers and cell bodies in central ganglia by moving the cobaltous chloride through the cut ends of axons in peripheral nerve trunks. I also used this method to stain "giant" fibers and other interneurons within the ganglia by moving the cobalt into the ventral nerve cord through the cut ends of central connectives. By the use of this technique, the course of fibers could be visualized traversing almost the entire length of the portion of the ventral nerve cord studied. In their study using the ventral nerve cord of Periplaneta americana, Pitman et al., (1973) were able to trace giant fibers a length of 2 cm and the range of this method may be even greater. In their original paper, Pitman et al., (1972) injected cobaltous chloride into a previously identified nerve cell body in an insect ganglion and after precipitation with ammonium sulfide the soma and its branches were darkly and clearly stained. This technique requires more elaborate equipment than the "simple" axonal iontophoresis technique used in my experiments, such as microelectrodes

for injection of cobalt, a variable current supply, and monitering devices. The method I chose, iontophoresis through cut nerve trunks, also works well for localizing central nerve cell bodies and frequently stains the dendritic branchings within the neuropil. The method is simple and requires no elaborate equipment. Additionally, one of the primary assets of this technique is that there is less extracellular leakage of cobalt solution compared to the microelectrode injection technique. This is most likely due to the fact that there is no imposed electrical current forcing, or "pulling", the cobalt solution along the neuron.

The reason cobaltous chloride moves along the axons during axonal iontophoresis (without an applied current) is still a mystery. Kater et al., (1973) and Mason (personal communication) suggest that cobalt moves up the axons by simple diffusion. Kater et al., (1973) also suggest another possible mechanism which involves currents generated by the cut nerves as a result of injury sustained during the initial cutting. These currents would be in the proper direction to move the positively-charged cobalt ions along the axon. This theory may be correct because it has been reported that negatively charged procion yellow will not fill neurons without an imposed current (Mulloney, 1973).

If no current is employed during axonal iontophoresis, Mason (personal communication) points out that it is essential

that the nerve to be filled be thoroughly cleaned and any excess moisture be removed. This drying seems to create extra osmotic pressure when the hypotonic cobalt solution is applied to the cut end of the nerve. She has found moisture removal to often be a pivotal step in getting good fills while working on the brain-retrocerebral neuroendocrine complex in the locust Schistocerca vaga as well as in her studies on the innervation of the optic lobes of rodents (Mason, 1973, and by personal communication).

Axonal iontophoresis and intracellular injection of cobalt has recently been used as a means to other ends. Once the neuronal morphology is visualized with iontophoretic or injection techniques, it can be used as a "map" for intracellular recordings with microelectrodes in the insect nervous system (Murphey, 1973). Mulloney (1973) has relied on the cobalt injection technique to assure that the microelectrodes have been placed in the proper soma by first filling the injection site and then precipitating in situ to visualize whether or not the particular cell in question has been "hit". Intracellular recordings can then be carried out with positive assurance that the recordings are being taken from the proper cell body. The effect on behavior of a single gene mutation in the cricket has been recently studied (Bentley, 1975) using intracellular injection cobalt techniques.

Axonal iontophoresis is always applied in the direction towards the brain, ganglia, or other similar nervous tissue. If the nerve trunk is filled in the direction of the muscle or organ that it supplies, the entire preparation will be precipitated when exposed to the ammonium sulfide solution because ammonium sulfide will precipitate metal chlorides other than cobalt (Pitman et al., 1972). Saktor (1965) points out that large amounts of iron are needed in the form of iron chloride and iron phosphate for the oxidation of alpha-glycerophosphate, one of the many metabolic processes within insect muscles. Copper is also an essential element in certain metabolic pathways and would thus be available, along with iron, for precipitation with the ammonium sulfide.

Axonal iontophoresis through cut nerve trunks requires great care during the course of the preparation. Petroleum jelly must be sealed tightly around the base of the cut nerve to avoid leakage onto surrounding tissue and the ganglion. The proper amount of time for complete filling of the nerve must be found by trial and error in order to maintain viability of the insect systems, avoid desiccation, and achieve the desired results. Nerve tissue is delicate yet flexible and nerves with a diameter of 10 μm may often be filled successfully if a good degree of dexterity is possessed.

CONCLUSIONS

Axonal iontophoresis proved to be a very practical and efficient method for filling a group of cells whose small size ruled out the use of microelectrodes and micropipettes and whose exact location is unknown. However, there are some drawbacks which must still be overcome. First, the stain tends to migrate into the extracellular space within a nerve, especially when an electrical current is applied. The correct length of time for complete filling of neurons must be determined experimentally because it may vary with the tissues under study. Also, it is difficult to reproducibly fill particular neurons when their axons are part of a large nerve containing many axons (Mulloney, 1973).

In this study the following nerves of the ventral nerve cord ganglia of Acheta domesticus were filled:

1) Nerve I of the third thoracic ganglion.
2) Nerve III of the third thoracic ganglion.

Also, the fifth, sixth, and seventh abdominal ganglia were filled successfully via the posterior pair of interganglionic connectives of the seventh abdominal ganglion.

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